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Abstract: Mycoplasma suis belongs to haemotrophic mycoplasmas (HMs) which cause infectious anaemia in a large variety of mammals. To date, no in vitro cultivation system for M. suis or other HMs has been established. We hypothesised that M. suis could grow in classical Mycoplasma media supplemented with nutrients (e.g. glucose, iron-binding proteins) which are naturally available from its host environment, the porcine blood. Blood from experimentally M. suis-infected pigs was used to inoculate either standard SP-4 Mycoplasma medium supplemented with iron-binding proteins (transferrin, haemin, and haemoglobin) or glucose-enriched Hayflick Mycoplasma medium. A quantitative M. suis-specific real-time PCR assay was applied to determine and quantify M. suis loads weekly during 12 week-incubation. The first 2 weeks after inoculation M. suis loads decreased remarkably and then persisted at a stationary level over the observation time of 12 weeks in iron-binding protein- or glucose supplemented media variants. Scanning electron microscopic analysis of liquid M. suis sub-cultures on Hayflick agar showed small, densely-packed microcolonies of irregular M. suis cells of reduced size (0.2-0.6 m) indicating nanotransformation. The partial 16S rDNA sequence of these cultured M. suis nanocells was 99.9% identical to M. suis. M. suis cells derived from liquid cultures interact in vitro with porcine erythrocytes by fibril-like structures. We conclude, that the modified Mycoplasma media used for M. suis cultivation are obviously unfavourable for growth but lead to culture persistence. M. suis adapt to inappropriate culture conditions by alteration into nanoforms.

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Nanotransformation of the haemotrophic *Mycoplasma suis* during *in vitro* cultivation attempts using modified cell free *Mycoplasma* media

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Abstract

Mycoplasma suis belongs to haemotrophic mycoplasmas (HMs) which cause infectious anaemia in a large variety of mammals. To date, no *in vitro* cultivation system for *M. suis* or other HMs has been established. We hypothesised that *M. suis* could grow in classical *Mycoplasma* media supplemented with nutrients (e.g. glucose, iron-binding proteins) which are naturally available from its host environment, the porcine blood.

Blood from experimentally *M. suis*-infected pigs was used to inoculate either standard SP-4 *Mycoplasma* medium supplemented with iron-binding proteins (transferrin, haemin, and haemoglobin) or glucose-enriched Hayflick *Mycoplasma* medium. A quantitative *M. suis*-specific real-time PCR assay was applied to determine and quantify *M. suis* loads weekly during 12 week-incubation. The first two weeks after inoculation *M. suis* loads decreased remarkably and then persisted at a stationary level over the observation time of 12 weeks in iron-binding protein- or glucose supplemented media variants. Scanning electron microscopic analysis of liquid *M. suis* sub-cultures on Hayflick agar showed small, densely-packed microcolonies of irregular *M. suis* cells of reduced size (0.2 - 0.6 µm) indicating nanotransformation. The partial 16S rDNA sequence of these cultured *M. suis* nanocells was 99.9% identical to *M. suis*. *M. suis* cells derived from liquid cultures interact *in vitro* with porcine erythrocytes by fibril-like structures. We conclude, that the modified *Mycoplasma* media used for *M. suis* cultivation are obviously unfavourable for growth but lead to culture persistence. *M. suis* adapt to inappropriate culture conditions by alteration into nanoforms.

Keywords: haemotrophic mycoplasma, culture, quantitative PCR, scanning electron microscopy, nanotransformation

1. Introduction

Mycoplasma suis is a member of the haemotrophic mycoplasmas (HMs) which attach to or invade host erythrocytes of many mammals (Hoelzle, 2008; Groebel et al., 2009). In all these animals HMs are considered host-specific and to cause acute or chronic anaemia. Currently, the zoonotic potential of HM infections is intensely discussed (Dos Santos et al., 2008; Hu et al., 2009; Bosnic et al., 2010; Sykes et al., 2010; Steer et al., 2011).

HMs are highly specialised bacteria with a high degree of host adaptation reflected by its specific cell tropism, persistent infection, immune modulation, and, finally, uncultivability *in vitro*. All attempts to cultivate HMs *in vitro* have failed so far. Only a short-term maintenance of *M. suis* has been described using a petri dish erythrocyte culture system (Nonaka et al., 1996). Therefore, to date, HM research relies on the propagation in splenectomised animals, a method connected with serious ethical concerns. Moreover, cultivation of bacteria is an important feature of microbiology since Robert Koch. Substantial amounts of pure bacteria are the precondition for the analyses of bacterial characteristics and would enable the development of strategies for therapy and prophylaxis of HM infections.

In this study we investigated the application of modified standard culture techniques for mycoplasmas for the cultivation of *M. suis*. Furthermore, we investigated the ultra-structure of *M. suis* by scanning electron microscopy (SEM) after sub-cultivation on agar plates, and studied the *in vitro* interaction of culture-derived *M. suis* cells with porcine erythrocyte.

2. Material and Methods

2.1 Animal experiments and blood collection

In this study a splenectomised *M. suis* pig model was used (Hoelzle et al., 2003; Hoelzle, 2008). Na-citrate anti-coagulated blood was drawn at maximum bacteraemia (>90% of the erythrocytes parasitized). For *in vitro* interaction studies erythrocytes from *M. suis*-negative pigs were taken. The animal experiments were performed with the approval of the Veterinary Office of Zurich, Switzerland, under the registration no 55/2007.

2.2 Mycoplasma suis *culture*

Standard SP-4 *Mycoplasma* medium (ATCC medium 988) was prepared using PPLO broth base (Becton Dickinson, Basel, Switzerland), tryptone (Becton Dickinson), 0.5% glucose (Sigma), 0.5x CMRL-1066, 0.35x yeast extract, 1x yeastolate (all from Invitrogen, Basel, Switzerland), 17% foetal calve serum (FCS; Oxoid, Basel, Switzerland) , and penicillin G (f. c. 1000 U/ml; Sigma). Porcine haemin, haemoglobin (both from Sigma) or transferrin (First Link LTD; U.K.) were added individually (f. c. 100 mg/l). Modified Hayflick *Mycoplasma* medium (Hayflick, 1965) was prepared using PPLO broth base and 30% *Mycoplasma* enrichment mixture (Becton Dickinson). Finally, 1% glucose and penicillin G (f. c. 1000 U/ml) were added. For the Hayflick agar plates PPLO agar base was used.

All *M. suis* liquid cultures experiments were performed in triplicate in 3 ml-shell vial tubes (10% CO_2 atmosphere, 37°C). Cultivation started on day 0 with the addition of 30 µl blood from *M. suis*-infected pigs containing 1.5 x 10^8 *M. suis*/ml. Samples were taken at day 0 and then weekly. Not-inoculated cultures served as negative controls. Sub-cultures on Hayflick agar plates were carried out from liquid cultures after 8 weeks of incubation. Agar plates were incubated for 8 weeks at 37 °C and 10% CO_2.

2.3 *Inoculation of porcine erythrocytes with cultured* M. suis

M. suis negative erythrocytes were washed three times in PBS (Sigma) and finally suspended in RPMI-1640 medium (Sigma) containing 10% FCS. Erythrocytes (2.5%) were mixed in a 1:1 ratio with *M. suis*-positive liquid SP-4 transferrin cultures (taken at week 8 p.i.) and incubated at 37°C for 5 days. Negative control erythrocytes were prepared using pure medium.

2.4 *PCR and sequencing*

Liquid cultures (3 ml) were centrifuged (20000 x g, 20 min), and the pellet was used to extract DNA (GenElute Bacterial Genomic DNA Kit; Sigma). To quantify the *M. suis* loads in culture a quantitative Light Cycler-PCR assay was performed (Hoelzle et al., 2007).

Growth on Hayflick agar plates was specified by HM-specific 16S rDNA PCR (Hoelzle et al., 2011). Thus, *M. suis* sub-cultures were completely scraped off a Hayflick agar plate, collected in PBS and subjected to DNA extraction and 16S rDNA amplification. PCR amplicons were sequenced and gene sequences were compared with GenBank entries using the FastA algorithm (http://www.bio.uzh.ch/)

2.5 Scanning electron microscopy (SEM)

Hayflick agar plates (8 weeks after inoculation) were fixed with 3% glutaraldehyde (GA) for 1 h. Small 0.5 cm agar pieces were incubated in 3% GA overnight. Then, fixed agar samples were contrasted in 1% osmium tetroxide and 3% potassium ferrocyanide (Sigma). After ethanol dehydration and critical point drying the samples were sputter-coated with 12 nm Pt/C particles and analysed with a Zeiss Supra 50 VP scanning electron microscope.

Inoculated and not-inoculated erythrocytes were fixed with 1% GA and settled on 10 nm carbon-coated cover slips using a Cytospin 2 (Shandon, Dako-Diagnostica, Zug, Switzerland) centrifuge. After the subsequent acetone dehydration and critical point drying the samples were further prepared and analysed by SEM as described above.

3. Results

3.1 Mycoplasma suis*-cultivation in liquid cultures*

To date, iron-acquisition systems used by HMs are unknown. Iron bound in the host's blood to haemin, haemoglobin or transferrin could be one major limiting factor for the growth of HMs in pure culture. Therefore, SP-4 medium supplemented with haemin, haemoglobin or transferrin, respectively, was inoculated with *M. suis* containing porcine blood (f.c. 5 x 10^4 *M. suis*/ml medium). The results of the qLC-PCR quantification of the culture aliquots taken weekly are shown in Figure 1A and Table 1. After inoculation a decrease in the *M. suis* loads from mean 1.1 x 10^4 *M. suis*/ml medium (week 0) to mean 4.02 x 10^2 (1 week p.i.), and mean 1.45 x 10^2 (week 2 p.i.) *M. suis*/ml medium was observed. From week 2 onwards the *M. suis* contents of the cultures remained on a stationary level of about 1 to 5 x 10^2 *M. suis*/ml

medium (mean value) until the end of the experiment (week 12 p.i.). No significant differences in the *M. suis* culture loads could be found between the different media approaches. Using SP-4 medium without iron supplements no *M. suis* could be detected from 3 weeks p.i. until the end of the experiment (Figure 1A, Table 1).

Previous *in vitro* studies with *M. suis*-infected blood revealed a high *M. suis* glucose consumption (Smith et al., 1990). In this study we used glucose-enriched Hayflick medium for the cultivation of *M. suis*. Inoculation, incubation and culture load quantification were performed as described above. The *M. suis* culture loads decreased rapidly from mean 6 x 10^3 on average (inoculation) to mean 9.4 x 10^2 (week 1 p.i.), and mean 1.3 x 10^2 (week 2 p.i.; Figure 1B). From week 2 onwards the *M. suis* contents of the cultures remained on a stationary level of 0.9 to 2.1 x 10^2 *M. suis*/ml medium (mean value) until the end of the experiment (week 12 p.i.). Cultures in Hayflick medium without glucose were PCR-negative from week 3 p.i. until the end of the experiment (Figure 1B, Table 1).

3.2 Ultrastructure of M. suis *on culture plates*

On *M. suis* inoculated Hayflick agar plates we found *M. suis* microcolonies of varied size of between 2 x 4 µm to 2 x 10 µm distributed over the whole agar plate surface (Figure 2). Within the microcolonies single *M. suis* organisms with a small cell size ranging between 200 to 600 nm in diameter appeared to be closely connected to and barely distinguishable from each other. The *M. suis* cells observed demonstrated irregular and round shapes. The 16S rDNA PCR sequence demonstrated 99.9% identity to the 16S rDNA sequence of *M. suis* (GenBank: FN984917). On Hayflick agar plates inoculated with *M. suis*-negative Hayflick medium no bacterial structures could be observed.

3.3 In vitro *interaction of culture-derived* M. suis *with porcine erythrocyte*

Ultrastructural analysis of *M. suis*-negative erythrocytes incubated with liquid *M. suis* cultures revealed single *M. suis* cells attached to erythrocytes (Fig. 3). The connection between erythrocyte and *M. suis* organisms seemed to be mediated by fibril-like extensions.

Not-inoculated erythrocytes incubated under the same osmotic and media conditions showed no structural changes.

4. Discussion

Despite a long known significance of haemotrophic mycoplasmas no *in vitro* cultivation system has been established so far (Hoelzle, 2008). To the best of our knowledge, to date no systematic experimental studies concerning HM propagation in cell-free media have been published. In the present study we attempted to propagate *M. suis in vitro* based initially on two working hypotheses: First, we claimed that iron-containing proteins i.e. haemin, haemoglobin or transferrin are essential for *in vitro* growth. Iron is frequently the limiting factor for bacterial growth. Other haemotrophic bacteria such as *Bartonella* are known to require erythrocytes or haemin for *in vitro* growth. In fact, *B. quintana* has the highest known haemin requirement for a bacterium (Myers et al., 1969; Carroll et al., 2000). In this study iron-supplementation of SP-4 medium induced culture persistence of *M. suis* on a stationary level over a period of 12 weeks but not noticeable growth. However, we are not able to conclude which iron-containing protein is used by *M. suis* for iron acquisition, as we found no significant differences between the haemin-, haemoglobin- or transferrin-containing media. Only the used SP-4 broth without iron-containing proteins revealed completely negative results as early as after three weeks of incubation. Recent analysis of the whole *M. suis* genome sequences indicated that haemin may be the most probable iron-supplying protein for *M. suis* as, similar to *Bartonella*, *M. suis* possesses a haemin-specific transporter (Sander et al., 2000; Oehlerking et al., 2011).

The second approach was based on the fact that life-threatening hypoglycaemia is a major complication of acute *M. suis*-infection. The *M. suis* metabolism is obviously dependent on glucose and the metabolic activity of *M. suis* is the most logical reason for the dramatic decrease of the glucose level in the blood (Nonaka et al., 1990; Smith et al., 1990). Furthermore, during maturation porcine erythrocytes lose their permeability for glucose, and do not utilise glucose as the primary energy source (Kim and Luthra, 1977). These facts

have now been further supported by the results of the *M. suis* whole genome sequencing indicating that *M. suis* generates ATP solely through glycolysis (Guimaraes et al., 2011; Oehlerking et al., 2011). The used glucose concentration corresponds approximately tenfold the physiological glucose concentration in the porcine blood (Kixmoller et al., 2006). Cultivation in this medium also revealed culture persistence of *M. suis* similar to what had been observed in the first approach. Since all culture experiments using Hayflick medium without additional glucose supplementation or SP-4 broth without iron-containing proteins were *M. suis*-negative as early as after three weeks of incubation it could be assumed that the found persistent *M. suis* loads are not residues of the inoculum.

Ultra-structural analyses of *M. suis* on agar plates revealed strong evidence that our cultivation approach induces a transformation of *M. suis* into nanoforms that has already been described for other mycoplasmas, i.e. *Acholeplasma laidlawii* and *M. gallisepticum* (Chernov et al., 2007; Demina et al., 2010). *M. suis* microcolonies were densely packed indicating that the *M. suis* detected were obviously not inoculum residues. This was further supported by the fact that no erythrocyte residues were detected by SEM. The morphology of the culture-derived *M. suis* cells within the microcolonies differed distinctly from *in vivo* found *M. suis* cells in size and shape: The *M. suis* nanoforms were irregular and more compact roundish. In *Acholeplasma laidlawii* and *M. gallisepticum* nanotransformation was induced as an adaptive response due to adverse growth conditions (e.g. deficient medium). In addition to the morphological changes *A. laidlawii* and *M. gallisepticum* nanoforms differed significantly from its vegetative forms in their protein profiles (Chernov et al., 2007; Demina et al., 2010). Obviously, standard media provided suboptimal growth conditions for *M. suis* which is reflected by the observed nanotransformation. However, viability and metabolic activity of nanotransformed *M. suis* e.g. glucose consumption was not determined and this should be goal of further studies. Interestingly, nanotransformed *M. suis* derived from liquid cultures could be found in connection with healthy porcine erythrocytes *in vitro.*

In conclusion, standard mycoplasma media used in this study obviously provides *M. suis* suboptimal growth conditions leading to a transformation into nanoforms. In future, the

comprehensive and comparative genome and proteome analyses of HMs will help to identify the essential medium components for the establishment of *in vitro* cultivation systems for its propagation.

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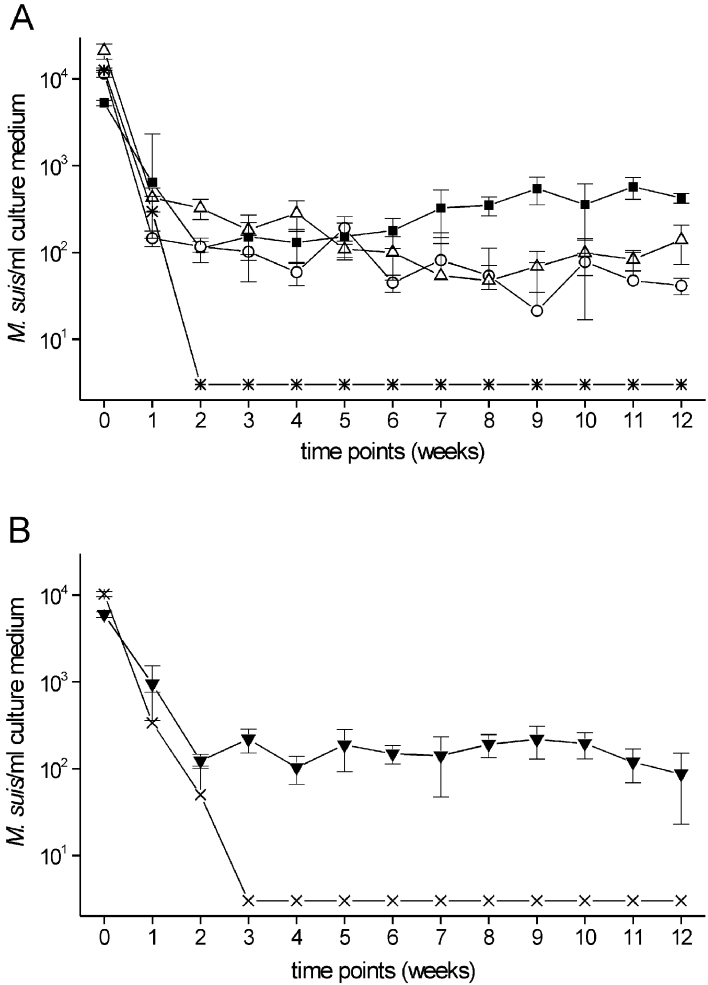
Figure legends

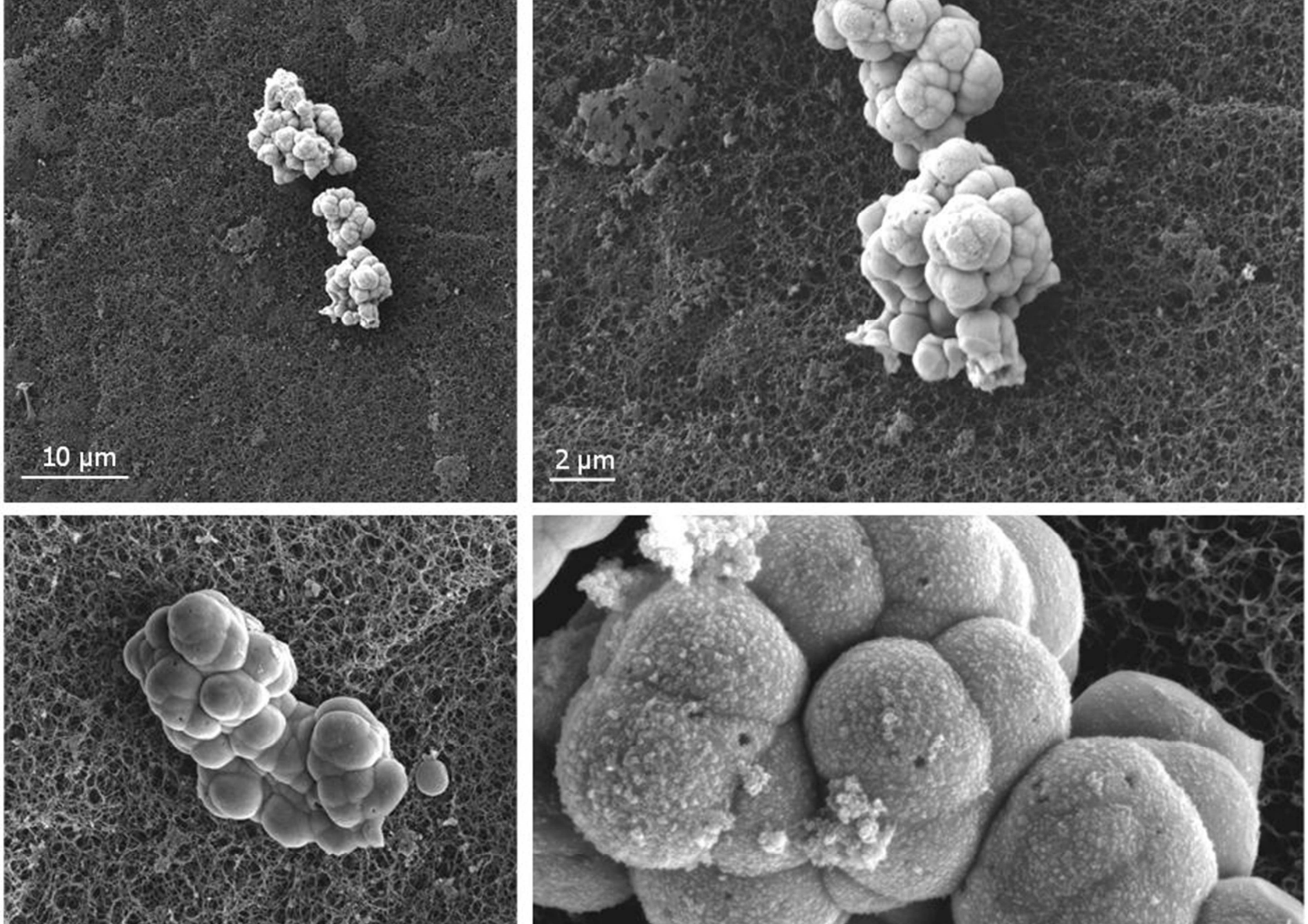
Fig 1. *M. suis* propagation in SP-4 and Hayflick *Mycoplasma* media. A: SP-4 medium (ATCC medium 988) supplemented with haemin (○), haemoglobin (Δ), transferrin (■) or without additional supplements (X) was inoculated using blood from an experimentally *M. suis* infected pig (f.c. 5 x 10^4 *M. suis*/ml medium). B: Hayflick medium supplemented with 1% glucose (■) or without additional glucose (X) was inoculated using blood from an experimentally *M. suis* infected pig (f.c. 5 x 10^4 *M. suis*/ml medium). Samples were taken weekly for 12 weeks. The *M. suis* loads were determined by a quantitative LC-PCR (Hoelzle et al., 2007).

Fig. 2 Scanning electron microscopy (SEM) of *M. suis* cultures grown on Hayflick-agar. *M. suis* liquid Hayflick cultures (8 weeks p.i.) were sub-cultivated on Hayflick agar plates. After 8 weeks of incubation agar pieces were fixed and analysed using SEM. *M. suis* microcolonies with varied size between 2 x 4 µm to 2 x 10 µm could be found on the agar surface. Within the microcolonies irregular roundish small *M. suis* cells ranging between 200 to 600 nm in diameter could be distinguished.

Fig. 3 Scanning electron microscopy (SEM) of porcine erythrocytes infected with culture-derived *M. suis*. Porcine erythrocytes were incubated with *M. suis*-positive liquid SP-4 transferrin cultures (taken at week 8 p.i.). After incubation for 5 days *M. suis* cells (arrows) attached to the surface of the red blood cells (RBCs) could be observed. Fibril-like extensions seem to mediate the contact between erythrocyte and *M. suis* organisms.

Figure 1


A
M. suis/ml culture medium
10^4
10^3
10^2
10^1
0 1 2 3 4 5 6 7 8 9 10 11 12
time points (weeks)
B
M. suis/ml culture medium
10^4
10^3
10^2
10^1
0 1 2 3 4 5 6 7 8 9 10 11 12
time points (weeks)


10 µm
2 µm

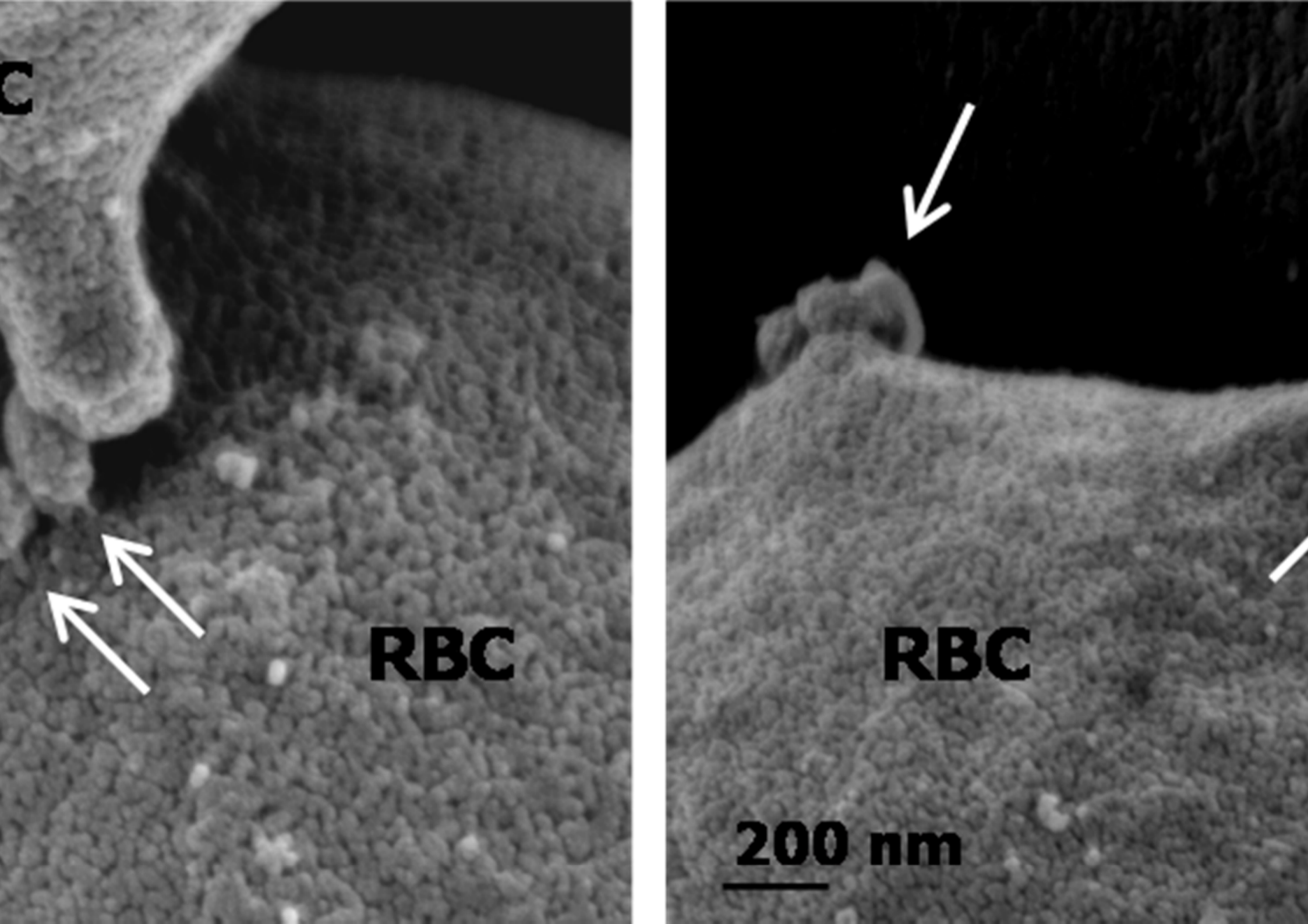

RBC
RBC
200 nm

Table 1

Mycoplasma suis culture approaches and qLC PCR quantification results.

Mycoplasma medium	Supplement	*M. suis* inoculation[C] (f.c. *M. suis*/ml medium)	qLC PCR result (*M. suis*/ml culture medium)			
			week 0	week 4	week 8	week 12
SP4	no supplements	5×10^4	1.3×10^4 ($\pm 7.7 \times 10^2$)	neg.	neg.	neg.
	haemin[A]	5×10^4	1.2×10^4 ($\pm 1 \times 10^3$)	6×10^1 ($\pm 2 \times 10^1$)	5×10^1 ($\pm 2 \times 10^1$)	4×10^1 ($\pm 1 \times 10^1$)
	haemoglobin[A]	5×10^4	2.1×10^4 ($\pm 4.2 \times 10^3$)	2.8×10^2 ($\pm 1.1 \times 10^2$)	4.7×10^2 ($\pm 6 \times 10^1$)	1.4×10^2 ($\pm 7 \times 10^1$)
	transferrin[A]	5×10^4	5.2×10^3 ($\pm 4 \times 10^2$)	1.3×10^2 ($\pm 0.6 \times 10^2$)	3.5×10^2 ($\pm 8 \times 10^1$)	4.2×10^2 ($\pm 5 \times 10^1$)
	transferrin[A]	-[D]	neg.	neg.	neg.	neg.
Hayflick	no supplements	5×10^4	1×10^4 ($\pm 7.2 \times 10^2$)	neg.	neg.	neg.
	glucose[B]	5×10^4	6×10^3 ($\pm 4.6 \times 10^2$)	1×10^2 ($\pm 4 \times 10^1$)	1.9×10^2 ($\pm 6 \times 10^1$)	9×10^1 ($\pm 6 \times 10^1$)
	glucose[B]	-[D]	neg.	neg.	neg.	neg.

A: f.c. 100 µg/ml medium; B: f.c. 10 mg/ml or 1%; C: blood from experimentally infected pigs was used as inoculum. D: negative control